Site-Directed Photosystem II Mutants with Perturbed Oxygen-Evolving Properties. 2. Increased Binding or Photooxidation of Manganese in the Absence of the Extrinsic 33-kDa Polypeptide *in Vivo*†

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ABSTRACT: Several site-directed photosystem II mutants with substitutions at Asp-170 or in the carboxy-terminal region of the D1 polypeptide were characterized *in vivo* in the absence of the extrinsic 33-kDa polypeptide. Site-directed mutations were constructed in the cyanobacterium *Synechocystis* sp. PCC 6803. The 33-kDa polypeptide was removed by insertional inactivation of the *Synechocystis psb*O gene. Mutants were characterized by measuring changes in the yield of variable chlorophyll *a* fluorescence following a saturating flash or brief illumination in the presence of an electron-transfer inhibitor or following each of a series of saturating flashes in the absence of inhibitor [Chu, H.-A., Nguyen, A. P., & Debus, R. J. (1994) *Biochemistry* (preceding paper in this issue)]. In the *presence* of the extrinsic 33-kDa polypeptide, many site-directed mutants contained a significant fraction of photosystem II reaction centers that lacked photooxidizable Mn ions. This fraction decreased dramatically in the *absence* of the extrinsic 33-kDa polypeptide, even in mutants having a significantly perturbed high-affinity Mn binding site (*e.g.*, in the mutants D170A and D170T). These results show that, *in vivo*, the extrinsic 33-kDa polypeptide directly or indirectly governs the occupancy of the high-affinity Mn binding site by Mn ions or the ability of bound Mn ions to reduce Y_Z^+.

The smallest oxygen-evolving photosystem II (PSII[1]) complex isolated to date contains at least eight subunits. The major subunits include an extrinsic 33-kDa polypeptide plus the membrane-spanning polypeptides CP47, CP43, D1, D2, the α and β polypeptides of cytochrome *b*-559, and the product of the *psb*I gene [for reviews, see Andersson and Styring (1991), Ikeuchi (1992), and Vermaas et al. (1993)]. The catalytic site of photosynthetic oxygen-evolution consists of a cluster of four manganese ions located in PSII near the lumenal surface of the thylakoid membrane. This cluster accumulates oxidizing equivalents in response to photochemical events within PSII and then oxidizes water by a mechanism that releases O_2 as a byproduct [for reviews, see Rutherford et al. (1992), Debus (1992), and Renger (1993)]. Chloride and 1–2 calcium ions are located near the manganese cluster and are required for catalysis. The extrinsic 33-kDa polypeptide covers, protects, and helps optimize the catalytic efficiency of the Mn cluster [for reviews, see Ikeuchi (1992), Debus (1992), and Barry et al. (1994)]. Cross-linking, accessibility, and other studies indicate that this polypeptide interacts with nearly all of the major PSII subunits [reviewed in Debus (1992)].

Instability of the oxygen-evolving apparatus in numerous site-directed cyanobacterial PSII mutants led us to further develop noninvasive methods for characterizing mutant PSII complexes *in vivo* (Chu et al., 1994). These methods involve measuring changes in the yield of chlorophyll *a* fluorescence after a single saturating flash or brief illumination in the presence of DCMU, or after each of a series of flashes in the absence of DCMU. By applying these methods to PSII mutants from which PSII particles had been isolated and characterized previously (*e.g.*, mutants at Asp-170 of the D1 polypeptide), we found that some PSII mutants, including some that evolve oxygen, contain a significant fraction of PSII reaction centers that lack photooxidizable Mn ions *in vivo* (Chu et al., 1994). In this fraction of reaction centers, either the high affinity Mn binding site from which Mn ions rapidly reduce Y_Z^+ is devoid of Mn ions or the Mn ion(s) bound at this site are unable to reduce Y_Z^+. These observations imply that, in such mutants, the Mn cluster is unstable or is assembled inefficiently *in vivo*.

In the present paper, we present a characterization of several site-directed PSII mutants in the absence of the extrinsic 33-kDa polypeptide. Removal of this polypeptide was accomplished by insertional inactivation of the *psb*O gene (Burnap & Sherman, 1991; Philbrick et al., 1991; Mayes et al., 1991; Bockholt et al., 1991). The site-directed mutations were constructed at Asp-170 or in the carboxy-terminal region of the D1 polypeptide in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Both Asp-170 (Nixon & Diner, 1992; Diner & Nixon, 1992; Boerner et al., 1992) and the carboxy-terminal residue (Ala-344) of the D1 polypeptide (Nixon et al., 1992) are essential for the stable assembly of a functional Mn cluster and may serve as ligands to the assembled cluster. Preliminary evidence similarly implicates His-332 and Asp-342 of the D1 polypeptide (Diner et al., 1991; Nixon et al., 1992; Chu et al., 1993). We found that the fraction of PSII reaction centers *without* photooxidizable Mn ions decreased dramatically in the absence of the extrinsic

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[1] Abbreviations: bp, basepair; Chl, chlorophyll *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_{eq}, steady-state fluorescence yield produced by weak monitoring flashes in the presence of DCMU; Gm^r, gentamycin resistant; kb, kilobase; MES, 2-morpholinoethanesulfonic acid; P_{680}, primary chlorophyll electron donor; PCR, polymerase chain reaction; PSII, photosystem II; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastoquinone electron acceptor; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; wild-type*, control *Synechocystis* strain constructed in the same manner as site-directed mutants, but with no mutation; Y_Z, rapid electron donor to P_{680}^+ (Tyr-161 of the D1 polypeptide); Y_D, slow electron donor to P_{680}^+ (Tyr-160 of the D2 polypeptide).

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33-kDa polypeptide. This was true even in mutants having a significantly perturbed high-affinity Mn binding site (*e.g.*, in the mutants D170A and D170T). We conclude that, *in vivo*, the extrinsic 33-kDa polypeptide directly or indirectly governs the occupancy of the high-affinity Mn binding site by Mn ions or the ability of the bound Mn ions to reduce Y_Z^+.

MATERIALS AND METHODS

Construction and Verification of Site-Directed Mutants. Construction of Asp-170 mutants was described previously (Boerner et al., 1992; Chu et al., 1994). The wild-type* strain was the same as in Chu et al. (1994) and was constructed identically to the Asp-170 mutants except that the transforming plasmid carried no mutation. To construct mutations in the carboxy-terminal region of the D1 polypeptide, the plasmid pAN1031 was created by ligating the 1.9 kb *Eco*RI/*Sma*I fragment of the *psb*A-2 vector pRD1031Km^r (Debus et al., 1988) into pUC119 (Vieira & Messing, 1987). Mutations were introduced into pAN1031 by methods described previously (Debus et al., 1988). To introduce the mutations into *Synechocystis* sp. PCC 6803, the 750 bp *Nco*I/*Xho*I fragment of pRD1031Km^r was replaced with the mutation-bearing 750 bp *Nco*I/*Xho*I fragment of pAN1031, and the resulting plasmid was transformed (Williams, 1988) into a strain of *Synechocystis* 6803 that lacks all three of its *psb*A genes (Debus et al., 1990). To confirm that homologous recombination had taken place, genomic DNA was isolated from each mutant, digested with *Xba*I, and analyzed by Southern blots (Debus et al., 1990). To confirm the identity of each mutant and to verify the absence of undesired mutations, a 1380-bp fragment of genomic DNA that encodes the entire *psb*A-2 gene was amplified by PCR, and the complete sequence of *psb*A-2 was obtained by direct sequencing of the double-stranded PCR product (Chu et al., 1994). For Southern analyses, genomic DNA was isolated by the procedure of Williams (1988) as modified by Chu et al. (1994). For sequence analyses, genomic DNA was isolated by the method of Chisholm (1989). To verify that no mutations outside the *psb*A-2 coding region contributed to the loss of photoautotrophy in the non-photoautotrophic carboxy-terminal mutants, cells of these mutants were transformed with a cloned fragment of the wild-type *psb*A-2 gene that included the desired mutation site. In every case, photoautotrophic transformants were recovered at frequencies that were far higher than the frequency at which spontaneous photoautotrophic revertants appeared. To verify that the silent mutations introduced to create or remove convenient sites for restriction endonucleases did not contribute to loss of photoautotrophy, spontaneous photoautotrophic revertants of non-photoautotrophic mutants were isolated and their *psb*A-2 genes amplified by PCR and sequenced (Chu et al., 1994). In every case in which the desired mutation had been created with a single base change, photoautotrophic cells were isolated in which the desired mutation had reverted (or a codon synonymous with the wild-type codon had been generated), but the silent mutation remained.

Insertional Inactivation of the psbO Gene. The plasmid pRB-1, which contains the entire *psb*O gene of *Synechocystis* sp. PCC 6803 on a 1.9-kb *Avr*II/*Hin*dIII fragment (Burnap & Sherman, 1991), was the generous gift of R. L. Burnap (Oklahoma State University). The plasmid pRZ1107, which contains a 2.0 kb *Bam*HI/*Bam*HI fragment conferring resistance to gentamycin (Yin et al., 1988), was the kind gift of C. P. Wolk (Michigan State University). To insertionally inactivate the *psb*O gene in site-directed D1 mutants and in the wild-type* strain, the Gm^r fragment of pRZ1107 was ligated into the *Bam*HI site of *psb*O in pRB-1 and the resulting plasmid was transformed into the mutant cells. Other workers have used this *Bam*HI site to insertionally inactivate *psb*O with DNA conferring resistance to kanamycin (Philbrick et al., 1991; Mayes et al., 1991; R. L. Burnap, personal communication). Transformants were selected on solid media containing gentamycin sulfate (10 μg/mL). To confirm segregation of homozygous Gm^r transformants, a fragment of genomic DNA bearing *psb*O (Philbrick & Zilinskas, 1988) was amplified by PCR using the oligonucleotides 5′-GTTCGCAAACAAACACCAACC-3′ and 5′-CTAAAAAGCCAGTCAGCTACC-3′. These oligonucleotides hybridize to complementary DNA strands approximately 230 bp upstream and 20 bp downstream of *psb*O, respectively. Amplification involved 25 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C in the reaction mixture recommended by Perkin-Elmer Cetus (Norwalk, CT). The size of the amplified fragment was ca. 3.1 kb in homozygous Gm^r transformants compared to 1.1 kb in wild-type cells (data not shown), confirming segregation. Southern blot analysis of genomic DNA isolated from homozygous transformants (Chu et al., 1994) also confirmed segregation, showing that the ca. 5 kb *Hin*dIII/*Hin*dIII fragment bearing *psb*O in wild-type cells had increased in size to approximately 7 kb (data not shown).

Other Procedures. Propagation and preparation of *Synechocystis* cells and measurements of light-saturated rates of oxygen evolution were performed as described previously (Chu et al., 1994). Measurements of chlorophyll *a* fluorescence were performed with a Walz (Effeltrich, Germany) pulse-amplitude-modulation fluorometer [described by Schreiber (1986)] as described previously (Chu et al., 1994). Weak monitoring flashes (λ = 650 nm) of 1 μs duration were applied at 1.6 or 100 kHz, as noted. For measurements of the fluorescence yield after each flash in a series, samples were incubated in darkness for 1 min before the weak monitoring flashes were switched on. The saturating actinic flashes were applied ca. 1 min later. For measurements of Q_A^- charge recombination kinetics after a flash or continuous illumination, samples were incubated in darkness for 1 min in the presence of 0.3 mM *p*-benzoquinone (purified by sublimation) and 1 mM potassium ferricyanide. DCMU was then added to a concentration of 40 μM (the final concentration of ethanol was 2%) and the weak monitoring flashes were switched on. In response to these flashes, the fluorescence rose from an initial level of F_0 to a steady-state level, denoted "F_{eq}". The ratio $(F_{eq}-F_0)/(F_{max}-F_0)$ ranges from ca. 0.25 in wild-type* cells to ca. 0.05 in mutants without photooxidizable Mn ions (Chu et al., 1994). Kinetics of Q_A^- oxidation were analyzed using Jandel Scientific's (San Rafael, CA) PeakFit program, version 3.18. Relative PSII contents were estimated from the difference between the initial fluorescence yield (F_0) measured by the monitoring flashes after 5 min of incubation in darkness with *p*-benzoquinone and potassium ferricyanide and the maximum fluorescence yield (F_{max}) produced by continuous actinic illumination in the presence of DCMU and 20 mM hydroxylamine (Chu et al., 1994).

RESULTS

Growth, Oxygen-Evolution Characteristics, and PSII Contents of Mutant Cells. The growth characteristics, light-saturated oxygen-evolution rates, and PSII contents of the mutant strains discussed in this paper are listed in Table 1. All of the mutant strains were obligate photoheterotrophs, requiring 5 mM glucose for propagation, except the wild-

Table 1: Comparison of Mutant Strains Whose Q_A^- Charge Recombination Kinetics Are Discussed

strain	O_2 evolution[a] (% of wt*)	PSII content[b] (% of wt*)	kinetics of Q_A^- oxidation after a single flash[c] (%)	k^{-1} (s)	no. cultures included in analyses
A344stop	0	104 ± 17	53 ± 5	0.015 ± 0.003	
			36 ± 5	0.28 ± 0.04	5
			12 ± 1.5	3.5 ± 0.6	
S345P	0	25 ± 3	29 ± 7	0.08 ± 0.06	
			32 ± 5	1.1 ± 0.3	3
			39 ± 8	8.3 ± 1.9	
A344stop-*psb*O	0	44 ± 5	27 ± 10	0.12 ± 0.06	
			22 ± 3	1.2 ± 0.9	5
			52 ± 12	8.8 ± 1.9	
wild-type*-*psb*O	32 ± 11	64 ± 7	20 ± 1	0.17 ± 0.04	
			38 ± 6	1.3 ± 0.3	3
			42 ± 7	8.2 ± 2.2	
D170H-*psb*O	0	31 ± 5	35 ± 2	0.20 ± 0.05	
			19 ± 6	1.3 ± 0.2	2
			47 ± 6	8.7 ± 0.4	
D170R-*psb*O	0	52 ± 14	25 ± 4	0.11 ± 0.07	
			13 ± 6	0.75 ± 0.35	2
			62 ± 3	8.1 ± 0.4	
H332N-*psb*O	0	30 ± 6	26 ± 4	0.09 ± 0.06	
			20 ± 9	0.9 ± 0.4	3
			54 ± 8	8.0 ± 0.9	
E333Q-*psb*O	0	35 ± 2	30 ± 8	0.07 ± 0.04	
			18 ± 10	0.9 ± 0.4	3
			51 ± 3	7.6 ± 0.1	
E333D-*psb*O	0	24 ± 3	29 ± 5	0.08 ± 0.02	
			21 ± 3	0.67 ± 0.43	3
			51 ± 7	7.9 ± 1.3	
E333H-*psb*O	0	50 ± 4	21 ± 2	0.09 ± 0.03	
			24 ± 4	1.0 ± 0.1	2
			55 ± 6	9.7 ± 1.4	
H337L-*psb*O	0	53 ± 3	26 ± 5	0.14 ± 0.05	
			14 ± 8	1.4 ± 0.9	3
			60 ± 15	8.6 ± 3.0	
D170A-*psb*O	0	48 ± 12	50 ± 7	0.022 ± 0.007	
			14 ± 2	0.32 ± 0.05	3
			39 ± 7	6.9 ± 0.8	
D170T-*psb*O	0	26 ± 3	41 ± 6	0.15 ± 0.02	
			11 ± 7	2.4 ± 1.7	2
			47 ± 10	9.3 ± 1.2	

[a] The eight wild-type* (wt*) cultures used in this study exhibited 680 ± 30 μmol O_2 (mg of Chl)$^{-1}$ h^{-1}. [b] Estimated from the total yield of variable chlorophyll *a* fluorescence ($F_{max} - F_0$). [c] Measured in the presence of DCMU and analyzed assuming three exponentially decaying components. The relative amplitude (%) and the inverse of the rate constant of each component are reported.

type*-*psb*O mutant, whose properties were the same as the original Δ*psb*O strain (Burnap & Sherman, 1991) [also see Table 1 in Chu et al. (1994)]. On the basis of the total yield of variable chlorophyll *a* fluorescence ($F_{max} - F_0$) at a constant chlorophyll concentration (Philbrick et al., 1991; Nixon & Diner, 1992; Nixon et al., 1992; Chu et al., 1994), A344stop and S345P cells contained ca. 100% and 25% the PSII content of the wild-type* cells. None of the mutants except wild-type* evolved oxygen in the absence of the extrinsic 33-kDa polypeptide. In the absence of the extrinsic 33-kDa polypeptide, the PSII content of A344stop cells decreased to ca. 44% (strain A344stop-*psb*O, Table 1). Indeed, deletion of the *psb*O gene decreased the PSII content of every mutant by 30–70%. For example, the PSII content of D170H, D170R, D170T, and D170A cells relative to wild-type* cells was 90–130% in the presence of the extrinsic 33-kDa polypeptide (Chu et al., 1994), but only 26–48% in its absence (Table 1). The characteristics of the A344stop and S345P mutants listed in Table 1 agree with previous characterizations of these mutants when constructed in the *psb*A-3 gene of *Synechocystis* 6803 (Nixon et al., 1992).

Charge Recombination between Q_A^- and PSII Electron Donors. The kinetics of charge recombination between Q_A^- and the donor side of PSII is sensitive to the presence or absence of photooxidizable Mn ions in PSII (Nixon & Diner, 1990, 1992; Boerner et al., 1992; Chu et al., 1994). The yield of variable chlorophyll *a* fluorescence is believed to be proportional to the concentration of Q_A^- in *Synechocystis* 6803 (Philbrick et al., 1991; Nixon et al., 1992). Consequently, the kinetics of charge recombination can be measured from the decay of fluorescence yield that follows a saturating flash given in the presence of DCMU. Representative data for the mutants A344stop and S345P are shown in Figure 1. The $(F_{eq} - F_0)/(F_{max} - F_0)$ ratios were ca. 0.11 in A344stop cells and 0.2–0.3 in S345P cells. The decay kinetics were analyzed assuming three exponentially decaying components (Chu et al., 1994). In A344stop cells (Figure 1A), the charge recombination kinetics were mostly rapid, exhibiting components of 15 ms (53%), 0.28 s (36%), and 3.5 s (12%). In S345P cells (Figure 1B), the charge recombination kinetics were mostly slow, exhibiting components of 80 ms (29%), 1.1 s (32%), and 8.3 s (39%). The charge recombination kinetics in S345P cells resembled those of wild-type* cells in the absence of the extrinsic 33-kDa polypeptide (wild-type*-*psb*O cells, Figure 1D). The latter cells exhibited components of 0.17 s (20%), 1.3 s (38%), and 8.2 s (42%) (Table 1).

In both the A344stop and S345P mutants (Figure 1A,B), the increase in fluorescence yield produced by a saturating flash (relative to F_{max}) was significantly lower than in wild-type* cells (not shown). However, in the presence of 20 mM

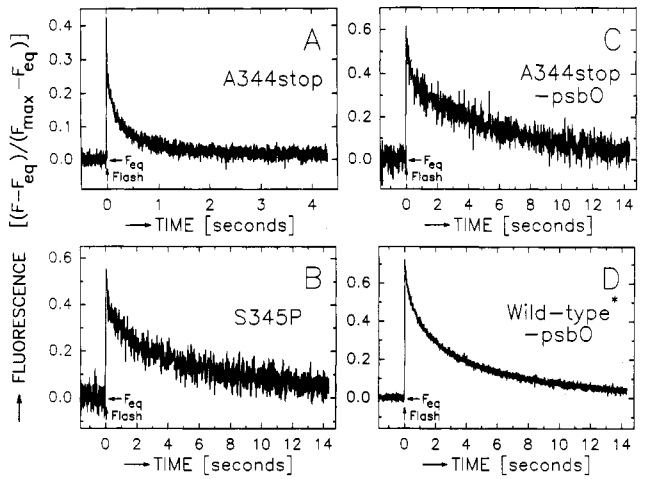

FIGURE 1: Charge recombination kinetics between Q_A^- and oxidized PSII electron donors in mutant cells of *Synechocystis* 6803 as measured by the decay of chlorophyll *a* fluorescence yield after a saturating flash given in the presence of DCMU: (A) A344stop, (B) S345P, (C) A344stop cells without the extrinsic 33-kDa polypeptide (A344stop-*psb*O), (D) wild-type* cells without the extrinsic 33-kDa polypeptide (wild-type*-*psb*O). Conditions: 20 μg of Chl in 0.58 mL of 50 mM MES–NaOH, 25 mM $CaCl_2$, 10 mM NaCl, pH = 6.5, 22 °C. Samples were incubated in darkness for 1 min in the presence of 0.3 mM *p*-benzoquinone and 1 mM potassium ferricyanide before DCMU was added to a concentration of 40 μM (the final concentration of ethanol was 2%). The monitoring flashes were applied at 1.6 kHz. The F_{eq} value is the steady-state fluorescence yield produced by the weak monitoring flashes in the presence of DCMU (see Materials and Methods). The F_{max} values were obtained by illuminating duplicate samples for 5 s (see below, Figure 3). The F_{max} values were not increased by the addition of hydroxylamine. Each trace represents the computer average of 9–16 traces. Note the different time scale in part A for the A344stop mutant.

hydroxylamine, the flash-induced increases in both mutants were slightly higher than in wild-type* cells (not shown). If the yield of the first flash in a series is denoted F_1, the ratio $(F_1 - F_0)/(F_{max} - F_0)$ was 0.73 ± 0.05 and 0.73 ± 0.02 for A344stop and S345P cells, respectively, compared to 0.59 ± 0.06 for wild-type* cells (Chu et al., 1994). These values show that the lower flash-induced yields in the absence of hydroxylamine (Figure 1) were not caused by slowed electron transfer from Y_Z to P_{680}^+ (Chu et al., 1994). An increased equilibrium concentration of P_{680}^+ may contribute to the lower flash-induced yield in A344stop cells in the absence of hydroxylamine. However, the similarity of the charge recombination kinetics of S345P and wild-type*-*psb*O cells suggests that, as in other wild-type*-*psb*O cells (Philbrick et al., 1991; Chu et al., 1994), a fluorescence quencher other than P_{680}^+ contributes to the lower flash-induced yield in S345P cells in the absence of hydroxylamine.

Quenching of Chlorophyll Fluorescence after the Second and Subsequent Flashes in a Series. The maximum yield of chlorophyll fluorescence produced by each flash in a closely-spaced series given in the absence of DCMU is also sensitive to the presence or absence of photooxidizable Mn ions in PSII (Nixon & Diner, 1990, 1992; Boerner et al., 1992; Chu et al., 1994). The highly fluorescent state $Y_Z^+P_{680}Q_A^-$ is formed 20 ns to 40 μs after the first flash, depending on whether or not the Mn cluster is present. The subsequent fluorescence decay represents the oxidation of Q_A^- by Q_B (Bowes & Crofts, 1980). If present, the Mn cluster reduces Y_Z^+ within 30–1300 μs (Dekker et al., 1984). However, if the Mn cluster is absent or not photooxidizable, Y_Z^+ is reduced by an alternate electron donor (possibly via P_{680}) at a slower rate. If a second saturating flash is given before Y_Z^+ has been reduced (but after Q_A^- has been oxidized by Q_B), the weakly fluorescent state

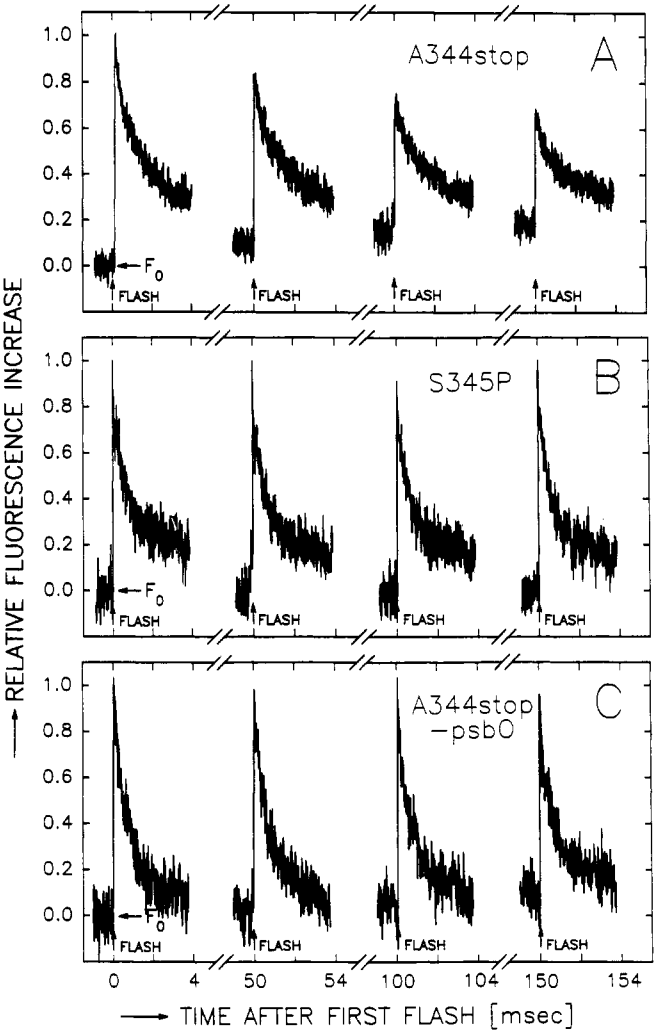

FIGURE 2: Yield of chlorophyll *a* fluorescence produced by each of four saturating flashes given at 50-ms intervals to mutant cells of *Synechocystis* 6803. Five milliseconds of data are shown for each flash: (A) A344stop, (B) S345P, (C) A344stop-*psb*O. The conditions were the same as in Figure 1 except that the cells were incubated in darkness for 1 min (without *p*-benzoquinone or ferricyanide) before the monitoring flashes were switched on, and no DCMU was added. The frequency of the monitoring flashes was switched from 1.6 to 100 kHz for 5 ms beginning 1 ms before each flash. The vertical scales were normalized to the maximum $(F - F_0)/F_0$ values observed after the first flash in each series. These values were 0.22 for A, 0.05 for B, and 0.05 for C. Six individual flash series were averaged for A, nine for B, and four for C.

$Y_Z^+P_{680}^+Q_A^-Q_B^-$ is formed. Consequently, the maximum fluorescence yield produced by the second flash will be lower than that produced by the first.

The fluorescence yields produced by each of four flashes given 50 ms apart to A344stop and S345P cells are shown in Figure 2, parts A and B, respectively. In A344stop cells (Figure 2A), the maximum fluorescence yields produced by the second and subsequent flashes were substantially quenched. These data show that the reduction of Y_Z^+ is slowed significantly in these cells, as expected for a mutant containing a significant fraction of reaction centers that lack of photooxidizable Mn ions (Chu et al., 1994). In contrast, little or no quenching was evident in S345P cells (Figure 2B). Wild-type* *psb*O cells also exhibited little or no quenching following the second or subsequent flashes (data not shown).

The rate of electron transfer from Q_A^- to Q_B is multiphasic [*e.g.* see Cao et al. (1991)]. In mutants without photooxidizable Mn ions (*e.g.*, D170A, D170N, and D170T), the

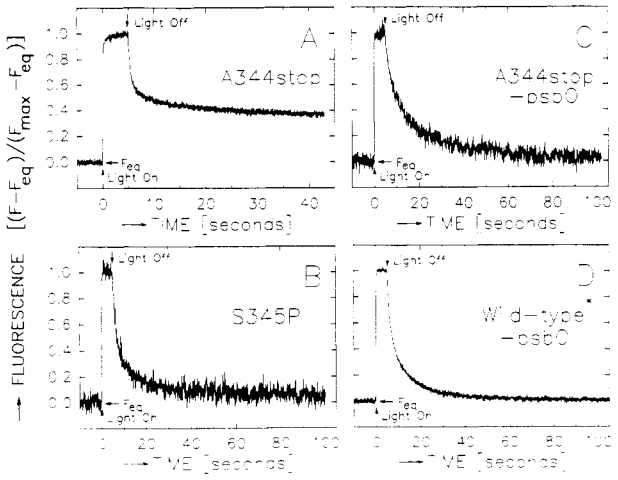

FIGURE 3: Formation and decay of Q_A^- in response to 5 s of continuous illumination in the presence of DCMU: (A) A344stop, (B) S345P, (C) A344stop-*psb*O, (D) wild-type*-*psb*O. The conditions were the same as in Figure 1. The onset and termination of illumination (controlled by a shutter that opened in 1.5 ms and closed in 3.0 ms) are indicated by arrows. Note the different time scale in part A for the A344stop mutant. For a definition of F_{eq}, see Materials and Methods. The monitoring flashes were applied at 1.6 kHz.

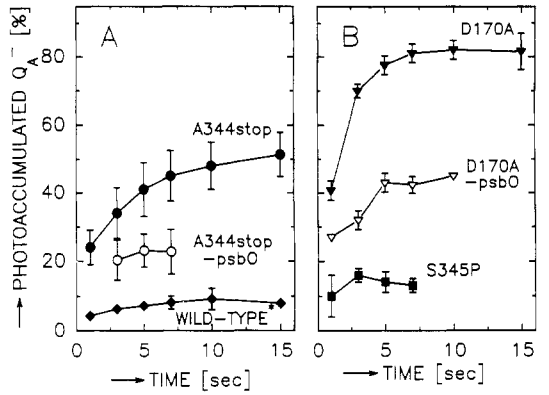

FIGURE 4: The fraction of PSII reaction centers that photoaccumulated Q_A^- after illumination for specific intervals of time. This fraction is defined as that fraction of PSII reaction centers in which Q_A^- is oxidized with a time constant of 1–2 min (see Figure 3 and text). (A) Photoaccumulation of Q_A^- in A344stop (top), A344stop-*psb*O (middle), and wild-type* (bottom) cells. (B) Photoaccumulation of Q_A^- in D170A (top), D170A-*psb*O (middle), and S345P (bottom) cells. The wild-type* and D170A data are reprinted from Chu et al. (1994) to facilitate comparisons.

slowest components of this electron transfer step are slower than in wild-type* cells, with significant fractions of Q_A^- remaining reduced 50 ms after each flash (Chu et al., 1994). Slowed electron transfer from Q_A^- to Q_B appears evident in A344stop cells (Figure 2A) but not in S345P cells (Figure 2B) nor in wild-type*-*psb*O cells (data not shown).

Photoaccumulation of Q_A^-. We have recently presented evidence that the fraction of reaction centers without photooxidizable Mn ions in site-directed PSII mutants can be estimated from the rate that Q_A^- photoaccumulates when cells are briefly illuminated in the presence of DCMU (Chu et al., 1994). To estimate the fractions of reaction centers lacking photooxidizable Mn ions in A344stop and S345P cells, cells of these mutants were illuminated for 1–15 s in the presence of DCMU. After illumination was terminated by a fast shutter, the kinetics of Q_A^- oxidation were analyzed assuming three exponentially decaying components (Chu et al., 1994). After 5 s of illumination, the Q_A^- oxidation kinetics in A344stop cells exhibited components of 0.38 s (44%), 3.5 s (16%), and 2 min (41%) (Figure 3A). In contrast, the Q_A^- oxidation kinetics in S345P cells exhibited components of 1.0 s (45%), 6.7 s (42%), and 2 min (14%) (Figure 3B). The Q_A^- oxidation kinetics of S345P cells resembled those of wild-type*-*psb*O cells (Figure 3D). The latter cells exhibited components of 1.2 s (48%), 9.0 s (48%), and 1.5 min (4%). These data show that photoaccumulation of Q_A^- is much more rapid in A344stop cells than in either S345P or wild-type*-*psb*O cells. Indeed, as shown in Figure 4A (upper curve), a significant fraction of PSII reaction centers in A344stop cells photoaccumulated Q_A^- during the first second of illumination. In contrast, S345P cells photoaccumulated little Q_A^- even after 7 s of illumination (Figure 4B, lower curve), similar to the extents of photoaccumulation in wild-type* cells (Figure 4A, bottom) and wild-type*-*psb*O cells (not shown).

Influence of the Extrinsic 33-kDa Polypeptide on the Fraction of Reaction Centers with Photooxidizable Mn Ions. The dramatically different behavior of A344stop and S345P cells in the experiments of Figures 1–4 was unexpected. Neither mutant assembles oxygen-evolving Mn clusters because the carboxy-terminal alanine residue of the D1 polypeptide is absent in the A344stop mutant and because the carboxy-terminal extension of the D1 polypeptide's precursor form is not posttranslationally cleaved in the S345P mutant (Nixon et al., 1992). In both mutants, the high-affinity site from which Mn ions rapidly reduce Y_Z^+ is largely intact, as determined from the K_M of electron donation from Mn^{2+} to Y_Z^+ in Mn-depleted PSII particles (Nixon et al., 1992). Because the high-affinity Mn binding site is largely intact in both mutants, we expected that both mutants would contain similar fractions of reaction centers with photooxidizable Mn ions. They do not. In A344stop cells, charge recombination after a single flash was mostly rapid (Figure 1A), the fluorescence yields produced by the second and subsequent flashes in a closely-spaced series were substantially quenched (Figure 2A), and a significant fraction of PSII reaction centers photoaccumulated Q_A^- during the first second of illumination (Figures 3A and 4A). These data imply that a significant fraction of PSII reaction centers in the A344stop mutant lack bound, photooxidizable Mn ions *in vivo*. In contrast, in S345P cells, charge recombination after a flash was mostly slow (Figure 1B), resembling that of wild-type* cells in the absence of extrinsic 33-kDa polypeptide (Figure 1D). In addition, the fluorescence yields produced by the second and subsequent flashes in a closely-spaced series were essentially unquenched (Figure 2B), the kinetics of Q_A^- oxidation after 5 s of illumination (Figure 3B) resembled those of wild-type* cells in the absence of the extrinsic 33-kDa polypeptide (Figure 3D), and little Q_A^- photoaccumulated, even after 7 s of illumination (Figures 3B and 4B). These data imply that, in contrast to the A344stop mutant, most PSII reaction centers in the S345P mutant contain photooxidizable Mn ions capable of rapidly reducing Y_Z^+.

Why should the fraction of PSII reaction centers without photooxidizable Mn ions be dramatically different in two mutants whose high-affinity Mn binding site is largely intact? Furthermore, why should the non-oxygen-evolving mutant S345P appear to contain a larger fraction of reaction centers with photooxidizable Mn ions than the photoautotrophic mutant D170H or the oxygen-evolving mutant D170R [*e.g.*, compare Figure 1B with Figure 2F,G of Chu et al. (1994)]? The similarity of the S345P mutant to wild-type* cells that lack the extrinsic 33-kDa polypeptide (*e.g.*, Figures 1B,D and 3B,D) led us to postulate that (1) the extrinsic 33-kDa polypeptide governs the occupancy of the high-affinity Mn binding site by Mn ions (or governs the ability of Mn ions in

this site to reduce Y_Z^+) and (2) the extrinsic 33-kDa polypeptide binds weakly or improperly to PSII in the S345P mutant. To test these hypotheses, we deleted the extrinsic 33-kDa polypeptide from several site-directed mutants by insertionally inactivating the *psb*O gene. The properties of our wild-type* strain in the absence of the extrinsic 33-kDa polypeptide were identical to those of the original Δ*psb*O mutant [*e.g.*, compare Figures 1D and 3D and the values in Table 1 in this work with Figures 2H and 5C and the values in Table 1 of Chu et al. (1994)].

Without the extrinsic 33-kDa polypeptide, charge recombination in the A344stop mutant slowed dramatically, from mostly rapid kinetics (Figure 1A) to mostly slow kinetics (A344stop-*psb*O, Figure 1C). The decay of fluorescence yield after a flash exhibited components of 0.12 s (27%), 1.2 s (22%), and 8.8 s (52%) (Table 1), values similar to those of the S345P and wild-type*-*psb*O mutants (Figure 1B,D; Table 1). In addition, the fluorescence yields produced by the second and subsequent flashes in a closely-spaced series were no longer quenched (Figure 2C), the slowest components of Q_A^- to Q_B electron transfer were accelerated to approximately wild-type rates (compare Figure 2A,C), and the kinetics of Q_A^- oxidation in A344stop-*psb*O cells after 5 s of illumination (Figure 3C) were similar to those of the S345P and wild-type*-*psb*O mutants (Figure 3B,D), exhibiting components of 1.4 s (29%), 9.9 s (48%), and 2 min (23%). Finally, the fraction of Q_A^- that photoaccumulated during illumination in the presence of DCMU decreased dramatically (Figure 4A, middle trace). These data indicate that, in A344stop cells, the fraction of PSII reaction centers without photooxidizable Mn ions decreased dramatically in the absence of the extrinsic 33-kDa polypeptide. In contrast, charge recombination in S345P cells was essentially unchanged in the absence of the extrinsic 33-kDa polypeptide (not shown).

Results similar to those obtained with A344stop cells were obtained when the extrinsic 33-kDa polypeptide was deleted from other mutants containing significant fractions of PSII reaction centers without photooxidizable Mn ions. Examples include D170H (Figure 5A,E), D170R (not shown), H332N (Figure 5B,F), E333Q (not shown), E333D (not shown), E333H (not shown), and H337L (not shown). In each of these mutants, the charge recombination kinetics after deletion of the extrinsic 33-kDa polypeptide resembled those of S345P, wild-type*-*psb*O, and A344stop-*psb*O cells (Table 1). Consequently, in each of these mutants, the fraction of reaction centers without photooxidizable Mn ions decreased dramatically in the absence of the extrinsic 33-kDa polypeptide.

The fraction of reaction centers without photooxidizable Mn ions also decreased dramatically when the extrinsic 33-kDa polypeptide was deleted from mutants having a significantly perturbed high-affinity Mn binding site. The PSII reaction centers in D170A and D170T cells lack photooxidizable Mn ions (Nixon & Diner, 1992; Boerner et al., 1992; Chu et al., 1994). Indeed, the K_M for the oxidation of Mn^{2+} by Y_Z^+ in isolated, Mn-depleted PSII particles from the D170A mutant is 50-fold higher than in Mn-depleted wild-type PSII particles (Nixon & Diner, 1992). Nevertheless, in the absence of the extrinsic 33-kDa polypeptide, the kinetics of charge recombination in cells of D170T (Figure 5C,G) and D170A (Figure 5D,H) slowed dramatically, with 39–47% of the fluorescence yield after a flash decaying with time constants of 7–9 s (Table 1). In addition, in D170A-*psb*O cells, the fraction of Q_A^- that photoaccumulated during illumination in the presence of DCMU decreased dramatically (Figure 4B, middle trace).

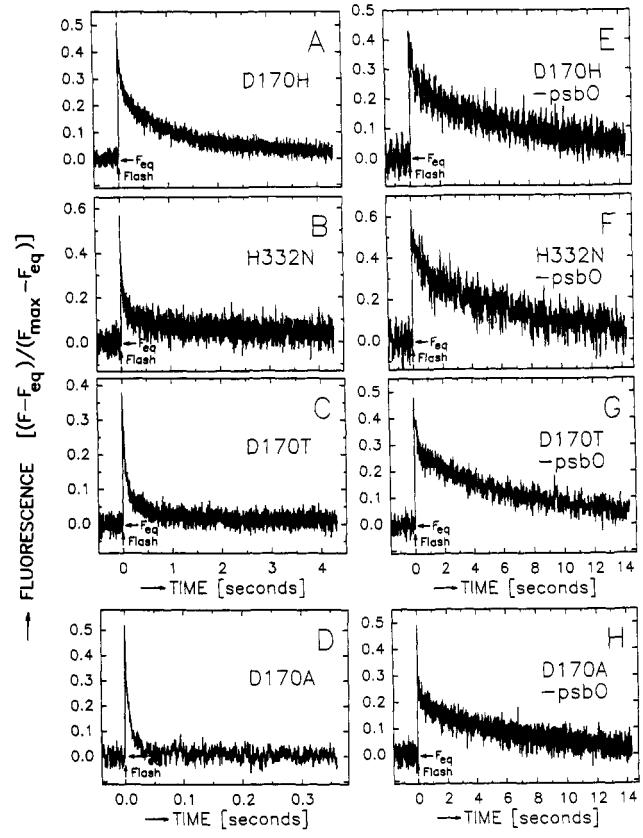

FIGURE 5: Charge recombination kinetics between Q_A^- and oxidized PSII electron donors in (A) D170H, (B) H332N, (C) D170T, (D) D170A, (E) D170H-*psb*O, (F) H332N-*psb*O, (G), D170T-*psb*O, (H) D170A-*psb*O cells. Note the three different time scales. The conditions were the same as in Figure 1. The traces in parts E, F, G, and H are the computer averages of 10–25 traces, while the other traces are the computer average of eight traces. The data for D170H (A), D170T (C), and D170A (D) were reprinted from Chu et al. (1994) to facilitate comparisons. For a definition of F_{eq}, see Materials and Methods. The monitoring flashes were applied at 1.6 kHz.

DISCUSSION

The data of Figures 1–4 show that a significant fraction of PSII reaction centers in A344stop cells lack photooxidizable Mn ions *in vivo*. By comparing the extent of Q_A^- photoaccumulation in A344stop cells with that in wild-type* and D170A cells after 10–15 s of illumination (Figure 4), one can estimate that 50–60% of the reaction centers in A344stop cells lack photooxidizable Mn ions *in vivo*. This fraction *decreased* to 10–25% in the *absence* of the extrinsic 33-kDa polypeptide (compare the top and middle traces of Figure 4A). Similarly, one can estimate that the fraction of reaction centers without photooxidizable Mn ions in D170A cells decreased from essentially 100% in the presence of the extrinsic 33-kDa polypeptide (Chu et al., 1994) to ca. 50% in the *absence* of this polypeptide (compare top and middle traces of Figure 4B). These estimates are in rough agreement with the fractions of Q_A^- that appear to recombine with Y_Z^+ after a single flash in A344stop and A344stop-*psb*O cells (Figure 1A,C; Table 1) and in D170A and D170A-*psb*O cells (Figure 5D,H; Table 1). However, these estimates should be considered to be very approximate for several reasons, as discussed previously (Chu et al., 1994): First, a fraction of reaction centers escapes detection in our experiments because of the actinic effect of our fluorescence monitoring flashes. On the basis of the measured $(F_{eq} - F_0)/(F_{max} - F_0)$ ratios, this fraction was ca. 11% in A344stop cells and 20–30% in S345P cells. Second, the rate of Q_A^- photoaccumulation varies considerably between mutants containing similar fractions of reaction centers with

photooxidizable Mn ions because of the dependence of the rate on the equilibrium concentration of P_{680}^+. Third, the multiphasic nature of charge recombination rates in PSII complicates attempts to assign specific recombination processes to specific rates.

In spite of the quantitation difficulties, the data of Figures 1–5 demonstrate that the extrinsic 33-kDa polypeptide governs (directly or indirectly) the occupancy of the high affinity Mn binding site by Mn ions or the ability of Mn ions bound at this site to reduce Y_Z^+. In the absence of the extrinsic 33-kDa polypeptide, the occupancy of this site by photooxidizable Mn ions increases dramatically, even in mutants whose high-affinity site is significantly perturbed (*e.g.*, in the D170T and D170A mutants).

The data of Figures 1–4 also show that most reaction centers in S345P cells contain photooxidizable Mn ions capable of rapidly reducing Y_Z^+. Furthermore, the acceleration of the slowest components of Q_A^- to Q_B electron transfer in S345P cells compared to A344stop cells (compare Figure 2A,B) suggests that a significant fraction of reaction centers in the S345P mutant may contain partially assembled Mn clusters [see the discussion in Chu et al. (1994)]. The similarity of the charge recombination kinetics of S345P cells (Figure 1B) to those of wild-type*-*psb*O cells (Figure 1D), A344stop-*psb*O cells (Figure 1C), D170H-*psb*O cells (Figure 5E), H332N-*psb*O cells (Figure 5F), and other mutants in the absence of the 33-kDa polypeptide strongly suggests that the extrinsic 33-kDa polypeptide binds weakly or improperly to the reaction centers of the S345P mutant. In view of the effect of deleting this polypeptide from A344stop, D170H, and H332N cells, the lack of properly bound extrinsic 33-kDa polypeptides in the S345P mutant would explain why significantly more reaction centers in this mutant contain photooxidizable Mn ions than in the A344stop mutant. Numerous site-directed carboxy-terminal mutants of *Synechocystis* 6803 exhibit fluorescence characteristics similar to those of the S345P mutant (H.-A. Chu, et al., manuscript in preparation). These data suggest that the extrinsic 33-kDa polypeptide binds weakly or improperly to PSII in these mutants and further suggest that this polypeptide interacts with the carboxy-terminal region of the D1 polypeptide.

Many properties of the extrinsic polypeptides of PSII differ between higher plants and cyanobacteria [for reviews, see Vermaas et al. (1993) and Barry et al. (1994)]. For example, the extrinsic 33-kDa polypeptide is required for photoautotrophic growth of the eukaryotic green alga *Chlamydomonas reinhardtii* (Mayfield et al., 1987; de Vitry et al., 1989) but not for photoautotrophic growth of the cyanobacteria *Synechocystis* sp. PCC 6803 (Burnap & Sherman, 1991; Philbrick et al., 1991; Mayes et al., 1991) or *Synechococcus* sp. PCC 7942 (Bockholt et al., 1991). Eukaryotic PSII complexes contain two extrinsic polypeptides of 24 and 17 kDa that regulate the Ca^{2+} and Cl^- requirements of oxygen evolution [for reviews, see Vermaas and Ikeuchi (1992) and Ikeuchi (1992)]. Cyanobacterial PSII complexes lack these polypeptides [*e.g.*, Stewart et al. (1985a) and Koike and Inoue (1985)]. Instead, they contain one extrinsic polypeptide of 9–12 kDa (Stewart et al., 1985b; Rolfe & Bendall, 1989; Shen et al., 1992; Shen & Inoue, 1993a) and another known as cytochrome *c*-550 (Shen & Inoue, 1993a,b; MacDonald et al., 1994). Extraction of the cyanobacterial extrinsic polypeptides requires treatment with Tris, while extraction of the eukaryotic extrinsic polypeptides requires less stringent treatments. In addition to possessing different extrinsic polypeptides, eukaryotic and cyanobacterial PSII complexes also differ in their cation requirements. Eukaryotes require Ca^{2+}, while cyanobacteria can substitute other cations such as Na^+ or Mg^{2+} [*e.g.*, see Shen et al. (1992) and Pauly et al. (1992) and reviews by Yocum (1991, 1992) and Debus (1992)]. In cyanobacteria, the cation requirement appears to be governed by the extrinsic 33-kDa polypeptide (Philbrick et al., 1991).

Given the above considerations, it is possible that the properties of the extrinsic 33-kDa polypeptide described in this study do not apply to organisms other than cyanobacteria. Two sets of earlier observations are relevant to this point. In the first set, Hoganson et al. (1989) investigated the influence of the extrinsic 33-kDa polypeptide on the reduction of Y_Z^+ by Mn^{2+} ions added to Mn-depleted PSII membranes from spinach. It was concluded that the extrinsic 33-kDa polypeptide does not hinder the diffusional access of Mn^{2+} ions to Y_Z^+ at pH = 6.0 (Hoganson et al., 1989). However, the conditions employed by Hoganson et al. were very different from those employed in the present study, making direct comparison of the results difficult. The second set of observations involves the LF-1 mutant of eukaryotic alga *Scenedesmus obliquus*. This mutant resembles the S345P mutant of *Synechocystis* 6803 in that both mutants fail to posttranslationally cleave the carboxy-terminal extension of the D1 polypeptide's precursor form (Diner et al., 1988; Taylor et al., 1988; Nixon et al., 1992). Because of this lack of processing, both mutants fail to assemble intact Mn clusters (Nixon et al., 1992). Whether or not the nonprocessed carboxy-terminal extension of the D1 polypeptide prevents the *proper* binding of the extrinsic 33 kDa polypeptide in the LF-1 mutant is not known. However, the *strength* of binding does not appear to be significantly diminished as judged from Coomassie Blue-stained lithium dodecylsulfate polyacrylamide gels of wild-type and LF-1 PSII membranes (Preston & Seibert, 1989). The 24- and 17-kDa polypeptides *are* weakly bound in the LF-1 mutant, however (Metz et al., 1985). This weakened binding could be caused by the absence of the Mn cluster in this mutant (Miyao & Murata, 1989; Kavelaki & Ghanotakis, 1991), by structural perturbations that result from failure to posttranslationally process the carboxy-terminal extension of the D1 polypeptide (Seibert et al., 1989; Preston & Seibert, 1989) or by a combination of both factors. Similar structural perturbations presumably exist in the PSII reaction centers of the S345P mutant of *Synechocystis* 6803. Considering that some of the properties of the extrinsic 24- and 17-kDa polypeptides in eukaryotes appear to have been acquired by the extrinsic 33-kDa polypeptide in cyanobacteria [*e.g.*, Philbrick et al. (1991)], these structural perturbations may interface with proper binding of the extrinsic 33-kDa polypeptide in the S345P mutant of *Synechocystis*.

Additional studies will be required to determine if the apparent influence the extrinsic 33-kDa polypeptide on the binding of photooxidizable Mn ions observed in the present study is unique to cyanobacteria. Additional studies will also be required to determine if structural perturbations interfere with the proper binding of the extrinsic 33-kDa polypeptide in the LF-1 mutant of *Scenedesmus obliquus* or in site-directed mutants of *eukaryotic* organisms having mutations in the carboxy-terminal region of the D1 polypeptide.

A recent reconstitution study demonstrated that the extrinsic 12-kDa polypeptide of the cyanobacterium *Synechococcus vulcanus* binds only partially to isolated PSII particles in the absence of the extrinsic 33-kDa polypeptide (Shen & Inoue, 1993a). An analogous 9–12-kDa polypeptide exists in *Synechocystis* 6803 [J.-R. Shen and Y. Inoue, cited in Vermaas et al. (1993)]. Consequently, the deletion of the extrinsic 33-kDa polypeptide from our site-directed *Synechocystis* mutants may have prevented the proper binding of a 9–12-

kDa extrinsic polypeptide. Therefore, we cannot exclude the possibility that loss of the 9–12-kDa polypeptide of *Synechocystis* 6803 contributes to, or is responsible for, the increased binding of photooxidizable Mn ions observed in the present study when the extrinsic 33-kDa polypeptide is removed. Additional studies will be required to clarify this issue.

Intriguingly, the profoundly different properties of the A344stop and S345P mutants observed in the present study were not observed earlier by Nixon et al. (1992). The reason might be the different fluorescence detection methods employed by our group and theirs. Nixon, Diner, and co-workers apply weak detection flashes at discrete times. Because they are applied at low frequency, these flashes generate no actinic effect. In contrast, the high frequency of our weak detection flashes (1.6 kHz) generates a significant actinic effect, leading to nonzero $(F_{eq} - F_0)/(F_{max} - F_0)$ values in the presence of DCMU (Chu et al., 1994). Consequently, in our samples, reaction centers undergo charge separation and recombination events prior to the firing of an actinic flash or to the onset of actinic illumination. We suggest that these events create a locally high concentration of Mn^{2+} ions near the high-affinity Mn binding site when the extrinsic 33-kDa polypeptide is absent or improperly bound. Possibly these Mn^{2+} ions bind to nonspecific sites that are inaccessible when the extrinsic 33-kDa polypeptide is properly bound.

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